THE CHEMISTRY OF ALLERGENS XX. New Antigens Generated by Pepsin Hydrolysis of Bovine Milk Proteins

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XX. New antigens generated by pepsin hydrolysis of bovine milk proteins

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Bovine serum albumin, α-lactalbumin, β-lactoglobulin, and casein were hydrolyzed at pH 2 with pepsin for 8 minutes. Hydrolyzates were separated into 2 fractions by dialysis—the dialysate and the endo fraction. Antigencity was determined by the Schultz-Dale technique with the use of uterine strips from guinea pigs sensitized with Freund's complete adjuvant. A new antigen was demonstrated in the dialysate of each of bovine serum albumin, α-lactalbumin, β-lactoglobulin, and casein. Casein was significantly less effective than the other proteins in the production of a new antigen. None of the antigens in the dialysates gave a precipitate with homologous rabbit antiserum. The endo fractions of β-lactoglobulin and casein contained no new antigens, and only one of 10 tests with the endo fraction of α-lactalbumin showed a new antigen. The endo fraction of bovine serum albumin contained a new antigen which gave a precipitate with homologous rabbit antiserum. Guinea pig antibodies for the dialysate of the pepsin digest of α-lactalbumin were stable when heated 4 hours at 56° C. These results may explain why milk proteins and possibly other foods in some cases do not give positive skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to this type of new antigen formed during digestion.

In this study the term "new antigen" is defined as an antigen with a specificity distinct from that of the protein from which it was generated. The purpose of this study was threefold: to simulate the first step in the digestion of bovine serum albumin (BSA), α-lactalbumin, β-lactoglobulin, and casein from bovine milk; to determine if new antigens were generated by brief pepsin hydrolysis of these proteins; and to determine some of the properties of the generated antigens. The results, herein reported, may explain why milk pro-

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teins and possibly other foods in many cases do not give skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to the new antigens formed during the first stages of digestion.

The immunologic significance of enzyme hydrolytic products of ingested allergenic proteins long has been the subject of speculation and investigation. The early concept was that only native proteins were capable of inducing immunological responses.[1] Landsteiner,[2] in 1945, stated that amino acids and low molecular weight peptides were inactive as antigens and that attempts to produce antibodies to relatively high molecular weight proteoses were generally unsuccessful.

Notwithstanding the foregoing viewpoint, sporadic reports appeared which indicated that protein cleavage products might have important immunologic and allergenic properties. Walker and associates[3] in 1923 observed 2 cases of positive skin reactions to artificial digests of foods when reaction to the unaltered foods gave negative skin tests. In 1942, Cooke[4] reported that 5 of 29 clinically sensitive patients tested gave positive skin reactions and passive transfer tests with proteoses from known sources, whereas the standard extracts of the corresponding unaltered foods gave negative skin tests. Other studies relevant to the allergenic significance of enzyme digests of foods have been reported by Urbach and co-workers,[5] Blamoutier,[6] and Bloom, Markow, and Redner.[7] Stull and Hampton[8] determined the antigenicity of primary and secondary proteoses prepared by a 4 day pepsin digest of various proteins with the use of the Schultz-Dale technique. Ishizaka and associates[9] obtained evidence that "hidden antigenic sites" were exposed by 24 hour pepsin hydrolysis at pH 4.2 of bovine serum albumin. However, these authors discarded the dialysate of their hydrolyzate, and they did not test for a new antigen with antiserum prepared with the hydrolyzate.

MATERIALS*

Bovine serum albumin

Crystalline bovine serum albumin from Pentex Corp., Kankakee, Ill., was used without further purification. The nitrogen content was 15.3 per cent.

Casein

Soluble or sodium casein was prepared from fresh skim milk as follows. To 2 L. of milk was added 720 Gm. of sodium chloride in increments with stirring at 28° C. Toluene was used as preservative throughout. After standing overnight, the precipitate was separated by centrifugation. The precipitate was washed twice with 400 and 600 ml. portions of saturated sodium chloride solution. The precipitate was then dispersed in 900 ml. of water and dialyzed at room temperature against 5 changes of 1N sodium chloride solutions for 6 days during which time the solid dissolved. The sodium chloride was then removed by dialysis against several 16 L. changes of water during 3 days. The opalescent

*The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

solution was filtered and lyophilized. The yield was 57 Gm. of solid containing 13.6 per cent nitrogen. Casein prepared in this way is known to contain some, if not all, of the "proteose-peptone" fraction of milk.[10] The sample contained no BSA, but both α-lactalbumin and β-lactoglobulin were detectable by gel diffusion analysis.

α-Lactalbumin

Purified α-lactalbumin from Pentex Corp. was used for further purification. Thirteen grams of α-lactalbumin was dissolved in 200 ml. of water. The solution, pH 7.0, was clarified by centrifugation and filtration. The pH of the solution was adjusted to 2.0 with 0.5N hydrochloric acid, and the precipitate was separated by centrifugation.* The supernatant solution was discarded, and the solid was washed twice with 200 ml. volumes of water adjusted to pH 2.0. The precipitate was suspended in 100 ml. of water and dissolved by the addition of 0.5N sodium hydroxide to pH 7.2. The solution on lyophilization yielded 5.4 Gm. of α-lactalbumin containing 11.6 per cent nitrogen. This sample did not contain any β-lactoglobulin as shown by gel diffusion analysis.

β-Lactoglobulin

Thrice recrystallized β-lactoglobulin from Pentex Corp. was used for further purification. Ten grams of β-lactoglobulin was dissolved in 100 ml. of 0.12N sodium chloride solution. The solution was clarified by centrifugation and then dialyzed for 7 days against several changes of water until free from sodium chloride. The precipitated β-lactoglobulin was recovered by centrifugation, washed once with 50 ml. of water, and dried in a vacuum over calcium chloride. The yield was 5.7 Gm. which contained 14.5 per cent nitrogen. This sample appeared to be pure β-lactoglobulin as shown by gel diffusion analysis and disc electrophoresis.

Dialysis tubing

The dialyzer tubing used retained materials with a molecular weight of 12,000 and higher.

Pepsin

Twice recrystallized swine pepsin was obtained from Worthington Biochemical Corp., Freehold, N. J.

EXPERIMENTAL

Pepsin hydrolysis

Five grams of protein was dissolved in 100 ml. of water. The solution was cooled in an ice bath, and an approximate amount of 0.5N hydrochloric acid was added to bring the pH near 2. The solution (or suspension in the case of α-lactalbumin) was then warmed rapidly in a water bath to 37 ± 1° C., and the pH was adjusted to 2.00. Five milliliters of a water solution containing 100 mg. of pepsin was added. The solution was maintained for 8 minutes at pH 2.00 ± 0.05 by dropwise addition of 0.5N hydrochloric acid while stirring. The hydroly-

*This sample precipitated at pH 2.0 because of the presence of residual sodium sulfate.

zate was then poured onto ice cubes and cooled to 5 to 8° C. in one minute to stop the reaction. A calculated amount of 0.5N sodium hydroxide was added to the ice-cold solution to neutralize the acid. The solution then was warmed to 25° C. and the pH adjusted to 7.5. The hydrolyzate was recovered by lyophilization.

Dialysis of pepsin hydrolyzate

The pepsin hydrolyzate from 5 Gm. of protein was dissolved in 50 ml. of water and dialyzed for 2 days against 500 ml. of water. The dialysis was continued with 2 more 500 ml. portions of water for 2 days each. The dialysates were combined and the dialysate (designated D) was isolated by lyophilization. The solution remaining inside the membrane was lyophilized to recover the endo fraction (designated E).

Schultz-Dale technique

Virgin, female guinea pigs, weighing about 225 grams, were sensitized by subcutaneous injections (nuchal area) with two 0.5 ml. volumes of the fraction emulsified with Freund's complete adjuvant. Dialysate fractions were dissolved in water and emulsified in a water-oil ratio of 1:1. Endo fractions were dissolved in physiological salt solution and emulsified in a water-oil ratio of 1:1.4. The sensitizing dose of dialysate contained 2 mg. of dialysate nitrogen. The sensitizing dose of endo fraction contained 5 mg. of solid. The incubation period was at least 28 days. Challenge doses were administered in terms of total nitrogen in the fraction. The basic Schultz-Dale technique with the use of uterine horns of the sensitized guinea pigs has been described previously.[11]

Rabbit antiserum

Rabbits were immunized by injection of 0.25 ml. of the fraction emulsified with Freund's complete adjuvant in each of the 4 footpads. The solvents and the water-oil ratios used for the emulsions were the same as described above. The immunizing dose of dialysate contained 2 mg. of dialysate nitrogen. The immunizing dose of endo fraction contained 5 mg. of solid. After an incubation period of 28 days, a single 1 ml. booster dose was administered intravenously. The booster dose of dialysate fractions contained 1 mg. of dialysate fraction nitrogen and that for the endo fractions contained 5 mg. of endo solid. Rabbits were bled out 7 days after administration of the booster dose.

Gel double-diffusion technique

The Ouchterlony[12] technique was used. Test and agar solutions were made up in 0.9 per cent saline buffered at pH 7.5 containing 0.01 per cent Merthiolate. A single filling of wells with antiserum and test solution was used. Results were read after 2 to 3 days.

Precipitin tests

Precipitin tests (ring and tube) were made with the use of twofold serial dilutions on a total nitrogen basis over the range of 1:1,000 through 1:32,000 for the dialysate fractions and corresponding original proteins.

The following abbreviations will be used to designate the fractions obtained by dialysis. The dialysates of the pepsin hydrolyzates of BSA, casein, α-lactalbumin and β-lactoglobulin are: BSAPD, CPD, LaPD, and LgPD, respectively. The corresponding endo fractions of BSA, casein, α-lactalbumin, and β-lactoglobulin are: BSAPE, CPE, LaPE, and LgPE, respectively.

RESULTS

Data pertinent to the pepsin hydrolysis and the dialysis of the hydrolyzates of the 4 milk proteins are shown in Table I.

Table I. *Data on pepsin hydrolyses and dialysis of pepsin hydrolyzates of milk proteins*

Protein	HCl per gram of protein* (mEq.)	Yield of dialysis fractions (Gm.)†		Nitrogen in dialysis fractions (% of total‡)	
		D§	E‖	D§	E‖
BSA	0.32	1.64	3.32	18.0	62.8
Casein	0.17	1.42	3.60	16.3	67.6
α-Lactalbumin	0.30	1.29	3.67	12.3	74.2
β-Lactoglobulin	0.11	0.76	4.3	5.6	82.2

*To maintain pH at 2.0 during hydrolysis.
†From 5.0 Gm. of protein, inclusive of sodium chloride formed.
‡Inclusive of pepsin nitrogen.
§Dialysate fraction.
‖Endo fraction.

Table II. *Response of the dialysates of the pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin and β-lactoglobulin in Schultz-Dale tests*

Protein	Sensitizing antigen*	Challenge dose of sensitizing antigen (μg of total nitrogen†)	Results		
			Animals tested (No.)	Positive for new antigen (No.)	Doubtful (No.)
BSA	BSAPD	10	5	2	0
	BSAPD	300	5	4	1
	None	300	4	0	0
Casein	CPD	10	8	0	2
	CPD	300	10	2	2
	None	300	3	0	1
α-Lactalbumin	LaPD	10	5	5	0
	LaPD	300	5	5	0
	None	300	4	0	0
β-Lactoglobulin	LgPD	10	5	5	0
	LgPD	300	5	5	0
	None	300	4	0	0

*The dialysate of the pepsin hydrolyzate of respective proteins.
†The ovarian halves of the 2 uterine horns from each animal were used separately, one for the 10 μg and one for the 300 μg challenge.

Table II contains a summary of the results of the Schultz-Dale tests for new antigens in the dialysates of the pepsin hydrolyzates of BSA, casein, α-lactalbumin, and β-lactoglobulin. All tests reported in Table II were conducted in the same manner as those illustrated in Figs. 1 to 4. Each of the dialysate fractions contained a new antigenic specificity as shown by these tests, although only 2 of 10 tests with casein elicited positive responses in contrast with much better responses with the other proteins.

Table III contains a summary of the results of Schultz-Dale tests for new antigens in the endo fractions obtained by dialysis of the pepsin hydrolyzates of the 4 proteins. The endo fraction from BSA contained a new antigen, and α-lactalbumin elicited one positive response in 5 tests. All tests with BSAPE were conducted in the same manner as that illustrated in Fig. 5 (Table III).

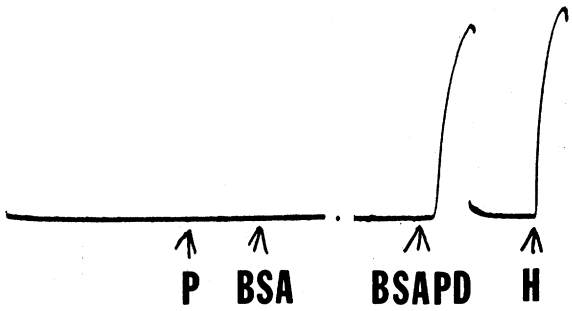

Fig. 1
Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *BSAPD,* of bovine serum albumin by the Schultz-Dale test. Sensitizing agent, *BSAPD.* Challenge dose in micrograms of total nitrogen: *P,* 100; *BSA,* 10; *BSAPD,* 10. (*H* = histamine.)

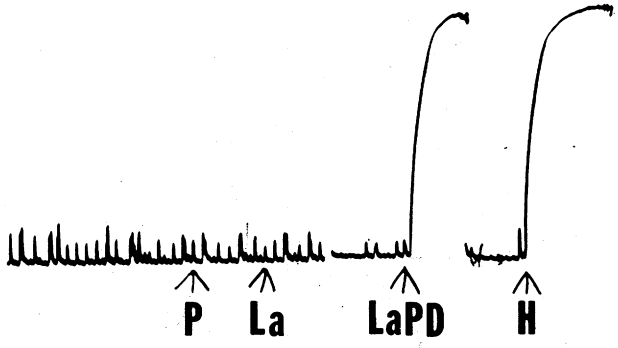

Fig. 2
Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LaPD,* of α-lactalbumin by the Schultz-Dale test. Sensitizing agent *LaPD.* Challenge dose in microgram of total nitrogen: *P,* 100; *La,* 10; *LaPD,* 10. (*H* = histamine.)

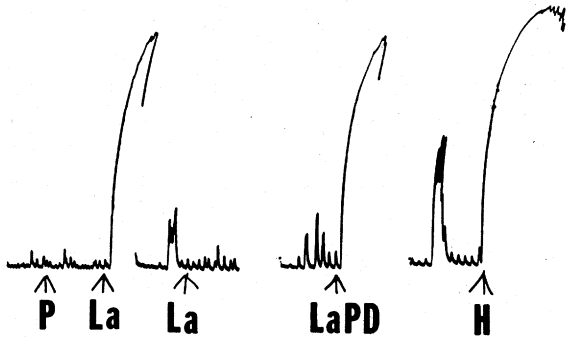

Fig. 3

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LaPD,* of α-lactalbumin in uterine strip which was also sensitive to α-lactalbumin by the Schultz-Dale test. Sensitizing agent, *LaPD.* Challenge dose in micrograms of total nitrogen: *P,* 100; *La,* 10; *La,* 10; *LaPD,* 10. (*H* = histamine.)

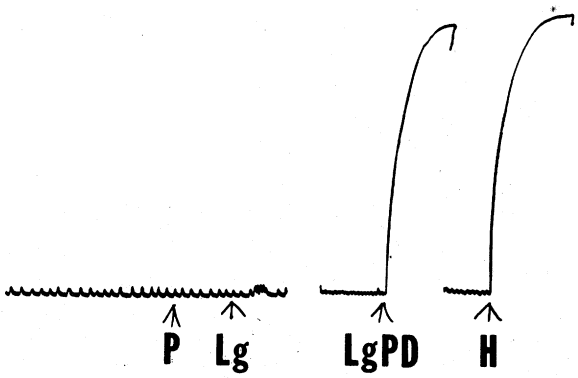

Fig. 4

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LgPD,* of β-lactoglobulin by the Schultz-Dale test. Sensitizing agent, *LgPD.* Challenge dose in micrograms of total nitrogen: *P,* 100; *Lg,* 10; *LgPD,* 10 (*H* = histamine.)

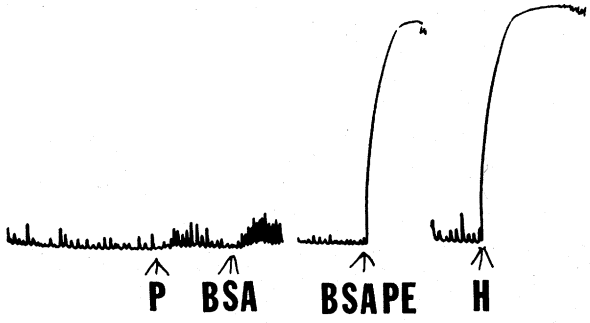

Fig. 5

Demonstration of a new antigen in the endo fraction of the pepsin *(P)* hydrolyzate, *BSAPE,* of bovine serum albumin by the Schultz-Dale test. Sensitizing agent, *BSAPE.* Challenge dose in micrograms total nitrogen: *P,* 100; *BSA,* 10; *BSAPE,* 10. (*H* = histamine.)

Table IV shows results of precipitin tests with the dialysate fractions, BSAPD, CPD, LaPD, and LgPD and the endo fraction, BSAPE, against their homologous antisera as compared with precipitin tests with the corresponding original proteins against the same antisera and of pepsin vs. anti-BSAPE. The dialysates did not precipitate, whereas BSAPE precipitated in high dilution.

Results of the gel double-diffusion tests showing the antigenic nonidentity

Table III. *Response of the endo fractions of the pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin, and β-lactoglobulin in Schultz-Dale tests*

Protein	*Sensitizing antigen**	*Challenge dose of sensitizing antigen (μg of total nitrogen†)*	*Results*		
			Animals tested (No.)	*Positive for new antigen (No.)*	*Doubtful (No.)*
BSA	BSAPE	10	4	4	0
	BSAPE	300	4	4	0
	None	300	4	0	0
Casein	CPE	10	4	0	0
	CPE	300	4	0	0
α-Lactalbumin	LaPE	10	5	1	1
	LaPE	300	5	1	1
β-Lactoglobulin	LgPE	10	4	0	0
	LgPE	300	5	0	0

*The endo fraction of the pepsin hydrolyzate of respective proteins.

†See footnote †, Table II.

Table IV. *Results of tests for precipitating antibody in rabbit antisera of dialysis fractions of pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin, and β-lactoglobulin*

Antiserum	*Test antigen*	*Precipitation titer* × 10-s*		
		Ring		*Precipitate (24 hours)*
		30 min.	*120 min.*	
BSAPD	BSAPD	0	0	0
	BSA	0	0	0
BSAPE	BSAPE†	1,024	2,048	512
	BSA	16	128	32
	Pepsin†	64	256	64
CPD	CPD	0	0	0
	Casein	0	0	0
LaPD	LaPD	0	0	0
	La	1	4	0
LgPD	LgPD	0	0	0‡
	Lg	±	1	0

*Highest dilution of antigen giving a precipitate, nitrogen basis. A zero reading indicates that all tests were negative over the range of dilutions of 1:1,000 through 1:32,000.

†Tests with dilutions of 1:1,000 through 1:128,000 were negative with normal rabbit serum.

‡A ± reading was obtained at 1:1,000 dilution only. This test was negative after 48 hours.

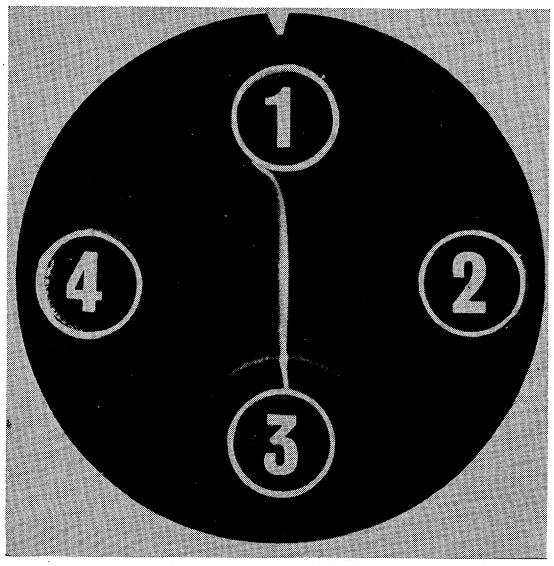

Fig. 6

Demonstration of new precipitating antigen from the endo fraction of the pepsin hydrolyzate, BSAPE, of bovine serum albumin by gel double diffusion analysis. Well *1*, 0.1 ml. of BSAPE, 0.4 mg. of nitrogen per milliliter; Well *2*, BSA, 0.1 ml., 0.05 mg. of nitrogen per milliliter; Well *3*, 0.1 ml. of BSAPE antiserum, Well *3* filled 24 hours before other wells were filled; Well *4*, 0.1 ml. BSA antiserum.

of BSAPE and BSA are shown in Fig. 6. Gel double-diffusion tests with the endo fractions CPE, LaPE, and LgPE with the use of homologous antisera showed that they retained the specificities of the original proteins and that no new specificities were present.

Guinea pig antibodies for LaPD, as an example, were homocytotropic as demonstrated by the PCA test. Guinea pig antiserum titers up to 1:80 were obtained. These antibodies were stable to heating for 4 hours at 56° C. These PCA tests were negative when challenged with α-lactalbumin in those guinea pigs giving a negative response to α-lactalbumin in the Schultz-Dale test, but the PCA tests were positive for α-lactalbumin in those guinea pigs giving a positive response with α-lactalbumin in the Schultz-Dale test.

DISCUSSION

The generation of low molecular weight antigens, possessing specificities distinct from those of the original proteins, by brief pepsin hydrolysis of 4 milk proteins has potential significance in food allergy (Figs. 1 to 4). This type of antigen may be the causative agent in rapid ($<$ 1 hour) clinical response to ingested milk, or possibly other foods, in persons not skin sensitive to the unaltered proteins. This type of antigen was not observed before because previous studies of enzyme-digested foods usually involved much longer times of digestion, the pH of digestion sometimes differed, dialyzable products were not isolated or were discarded, and/or methods of detection were not suitable.

In 1953, Bloom and associates[7] discussed the earlier work and the consensus of the clinical significance of enzyme-generated antigens and allergens. Although opinion was divided, the majority believed that digestive products caused delayed clinical responses of from over one hour to days. These authors used a 24 hour pepsin digest and a 24 hour pepsin digest followed by 24 hours digestion with trypsin for skin testing. Of a total of 268 feeding trials with unaltered foods, they found 2 cases (cocoa and wheat) in which asthma occurred within 30 minutes after feeding. In both cases the skin reaction was negative to the unaltered food but positive to the digests. Although clinical and cutaneous responses of these 2 cases conform to the hypothetical behavior of our new antigens, speculative comparison of similarity of their antigens and ours is not warranted because of the different times of digestion and the separation by dialysis of our antigens.

Owing to the complexity of protein molecules and the influence of various hydrolytic conditions on the splitting of these molecules, many new antigens could be generated during digestion. In preliminary experiments with total milk proteins, new antigens were detected in the dialysates after only 1, 2, and 4 minutes of pepsin hydrolysis. That these new antigens could be generated so rapidly could account for the immediate allergic response to ingestion of foods which in the unaltered state either do or do not react on the skin. It is well known that immunologically significant amounts of ingested allergen from peanuts, eggs, fish, cottonseed, and others are absorbed from the digestive tract in 5 to 30 minutes and that maximum absorption of allergen occurs within 2 hours.[13-15] That rapid absorption of allergen could occur directly from the stomach was demonstrated by Harten and colleagues.[16] These authors, using a passive transfer technique on rhesus monkeys, injected cottonseed allergen with a syringe directly through the wall into the lumen of the stomach which was clamped and sectioned at both the pyloroduodenal and cardioesophageal junctions. The sensitized cutaneous sites on the monkeys reacted in 8 to 18 minutes.

The immunological nonidentity of the dialysate antigens from α-lactalbumin and β-lactoglobulin was demonstrated by the Schultz-Dale technique. The uterine strip of a guinea pig sensitized with LaPD did not contract when challenged with LgPD but did contract when subsequently challenged with LaPD.

None of the new antigens gave nonspecific reactions with nonsensitized guinea pig uteri, as shown in Tables II and III.

No detectable amounts of bovine serum albumin and casein and very small amounts of α-lactalbumin and β-lactoglobulin passed into respective dialysate fractions as shown by the precipitin tests in Table IV with the use of rabbit antiserum prepared with the dialysate fractions. Traces of original protein in the dialysates occasionally sensitized guinea pigs. In such instances, however, the new specificity was easily demonstrated after desensitization of the uterine strip with the original protein (Fig. 3).

Pepsin in the dialysate fractions seldom sensitized the uterine strips. But strips were uniformly tested for sensitivity to pepsin. If this test was positive, the strips were desensitized to pepsin before we proceeded to the other tests.

Sensitization of guinea pigs to the new dialysate antigens with the use of the undialyzed pepsin hydrolyzate was difficult if not impossible. It was only after the separated dialysates were used for sensitization that uniform sensitivity was obtained.

In gel double-diffusion tests, the specificities of the original proteins were evident in the endo fractions when tested against a mixture of original protein antiserum and corresponding endo fraction antiserum, with the exception of BSAPE.

Of the 4 proteins studied, bovine serum albumin was unique in that the endo fraction contained a new precipitating antigen, BSAPE, as shown in the Schultz-Dale test (Fig. 5) and the gel diffusion test (Fig. 6). Although the line of precipitate of BSAPE (Fig. 6) was not heavy, nevertheless the precipitating titer of BSAPE with BSAPE antiserum was 1:2,048,000 compared with a titer of 1:128,000 for BSA with BSAPE antiserum (Table IV). Bovine serum albumin gave barely discernible lines of precipitate between Wells 2 and 3 vs. BSAPE antiserum, but BSA gave a very heavy line of precipitate against BSA antiserum as shown between Wells 2 and 4 (Fig. 6). It is apparent that the brief pepsin hydrolysis of BSA destroyed a major part of the original antigenic specificity of BSA. Pepsin substituted for BSAPE in gel diffusion analysis gave no line of precipitate (Fig. 6).

Further purification, chemical characterization, and clinical and immunological evaluation of these new antigens are planned for the future. It is recognized, however, that many new antigens may be generated by pepsin hydrolysis of proteins depending on such factors as time of hydrolysis, pH, temperature, pepsin sample used, and the like. Hence, in vitro–generated new antigens may not give positive skin reactions on any or all persons who are in fact sensitive to the product of their individual digestive systems. The value of this work is the demonstration that new, low molecular weight antigens are developed by brief pepsin hydrolysis of proteins.

Decision regarding the possible relationship of our new antigens from BSA and the "hidden antigenic determinants" of BSA suggested by Ishizaka, Campbell, and Ishizaka[9] was not in the scope of this investigation. Differences both in preparation and immunological demonstration and evaluation of their and our antigens indicate that they are different on the basis of available data.

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